AWARD NUMBER: W81XWH-16-1-0624

TITLE: Overcoming CRPC Treatment Resistance via Novel Dual AKR1C3 Targeting

PRINCIPAL INVESTIGATOR: Christopher P Evans MD

CONTRACTING ORGANIZATION: University of California, Davis, Davis, CA 95618

REPORT DATE: October 2017

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2017	Annual	30 Sep 2016 - 29 Sep 2017

4. TITLE AND SUBTITLE
Overcoming CRPC Treatment Resistance via Novel Dual AKR1C3 Targeting

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-16-1-0624

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Christopher P Evans

Email: cpevans@ucdavis.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
UNIVERSITY OF CALIFORNIA, DAVIS
1850 RESEARCH PARK DR, STE 300
DAVIS CA 95618-6134

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
AKR1C3 is the major AKR1C isozyme expressed in the human prostate; and elevated expression of this enzyme has been associated with prostate cancer progression and aggressiveness. Our hypothesis is that targeting AKR1C3 decreases intracrine androgens and AR variants and improves enzalutamide therapy against metastatic CRPC. During the first funding year, we demonstrated that AKR1C3 affected intracrine androgen biosynthesis. We introduced constructs expressing AKR1C3 under the control of doxycycline into LNCaP cells. We validated the biological function of the tet-inducible ARK1C3 in LNCaP/TR/AKR1C3 cells. When the gene was not induced, cell growth was readily inhibited by anti-androgens abiraterone (ABI) and enzalutamide (Enza). With doxycycline induction, the control treatment would benefit from overexpression of AKR1C3 and partially overcome the ABI and Enza inhibition. We measured intracellular and intratumor androgen levels by LC-MS induced by AKR1C3 and found that AKR1C3 expression induces intracrine androgen synthesis. We also demonstrated that overexpression of AKR1C3 in orthotopic model of LNCaP-AKR1C3 tumor confers resistance to enzalutamide treatment. These studies support the roles of AKR1C3 in intracrine androgen synthesis and confers resistance to enzalutamide.

15. SUBJECT TERMS
AKR1C3, intracrine androgens, enzalutamide, resistance

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	10	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

TABLE OF CONTENTS

1. INTRODUCTION:

Aldo-keto reductase family 1 member C3 (AKR1C3), also named 17BHSD5, is one of the most important genes involved in androgen synthesis and metabolism. AKR1C3 facilitates the conversion of weak androgens androstenedione (A' dione) and 5 α- androstanedione (5α-dione) to the more active androgens, testosterone and DHT respectively, which cannot be inhibited by abiraterone. It catalyzes steroids conversion and modulates steroid receptors trans-activation. AKR1C3 is the major AKR1C isozyme expressed in the human prostate; and elevated expression of this enzyme has been associated with PCa progression and aggressiveness. We have demonstrated that AKR1C3 was up-regulation in anti-androgen resistant prostate cancer cells. This overexpression conferred resistance to enzalutamide and was reversible by either AKR1C3 inhibitor or RNA interference. Our hypothesis is that targeting AKR1C3 decreases intracrine androgens and AR variants and improves enzalutamide therapy against metastatic CRPC.

2. KEYWORDS:

Androgen synthesis pathways; tet-inducible AKR1C3 expression; steroid measurement by LC-MS analysis, Abiraterone resistance, orthotopic animal model

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1 To determine the mechanisms of AKR1C3-mediated resistance to enzalutamide	**Timeline**	**Site 1**
Major Task 1: AKR1C3 affects intracrine androgen biosynthesis	Months	
Subtask 1: Generation of tet-inducible system that expresses AKR1C3 in LNCaP and C4-2B cells.	1-3	Drs. Gao, Evans
Subtask 2: Test the effect of controlled expression of AKR1C3 on intracrine androgens in LNCaP and C4-2B cells in vivo. *Two subcutaneous tumor models (LNCaP and C4-2B) will allow to develop over 10 weeks. Two groups (10x2 x2=40 mice) from each tumor xenograft models will be established.*	3-6	Drs. Gao, Evans
Subtask 3: Measuring intratumor androgen levels by LC-MS in AKR1C3 induced LNCaP and C4-2B tumor xenografts.	6-12	Drs. Gao, Evans
Milestone(s) Achieved: Overexpression AKR1C3 induces increases intracrine androgen levels	12	Drs. Gao, Evans

What was accomplished under these goals?

During the first funding year, our task was to validate that **AKR1C3 affected intracrine androgen biosynthesis**. There are three known androgen metabolism pathways so far. In the classical pathway, cholesterols are converted into DHEA and then androstenedione, which then to testosterone by AKR1C3. In some studies showed that androstenedione is preferentially metabolized into 5'-dione, and then to dihydrotestosterone (DHT) by AKR1C3. The lately proposed backdoor pathway used for intracrine androgen synthesis suggests that steroid will be produced through androsterone and to androstenediol by AKR1C3 to reach DHT. AKR1C3 is essential in all three routes (**Figure 1**). Levels of AKR1C3 vary among prostate cancer cell lines. Two of the castration resistance prostate cancer (CRPC) lines, CWR22Rv1 and VCaP cells express high levels of endogenous AKR1C3; whereas LNCaP cells and the subline LNCaP C42B with low or sub-detectable levels.

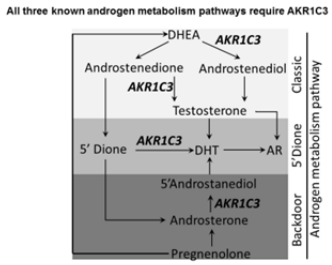


Figure 1. AKR1C3 is involved in the canonical, 5-diol, and backdoor pathways of androgen synthesis and activates AR.

Generation of tet-inducible AKR1C3 expression in LNCaP cells (led by Dr. Gao and assisted by Dr. Evans' labs) We therefore introduced constructs expressing AKR1C3 both alone or under the control of doxycycline into LNCaP cells, and selected for stable clones with respective antibiotics. Here we showed the characterization of the tet-inducible LNCaP/TR/AKR1C3 stale clone. With doxycycline in the dose of 1, 2.5 and 5 ng/ml treatments overnight, expression of AKR1C3 was induced in a dose-response manner (**Figure 2A**). At the same time, detection of AR-V7 and variants in general was apparent compared to the un-induced control. This observation is in agreement with our previous report that knocking down AKR1C3 in CWR22Rv1 and C4-2B-MDVR cells diminished AR-V7 expression (ref). We further validated the biological function of the tet-inducible ARK1C3 in LNCaP/TR/AKR1C3 cells. When the gene was not induced, cell growth was readily inhibited by anti-androgens abiraterone (ABI) and enzalutamide (Enza). Addition of androgen synthesis intermediates for two of the pathways, 5α-dione or androstenedione would bypass the inhibition elicited by ABI (on CYP17A1) but not much by Enza, suggesting some leakiness of the tet-responsiveness. With doxycycline induction, the control treatment

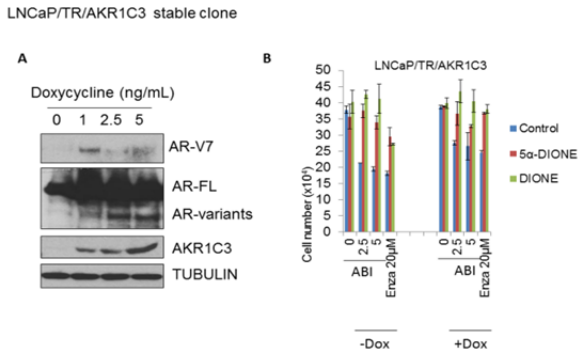


Figure 2. A. Validation of LNCaP/TR/AKR1C3 cells. B. Growth response of LNCaP/TR/AKR1C3 cells to stimuli of intermediates from androgen synthetic pathway.

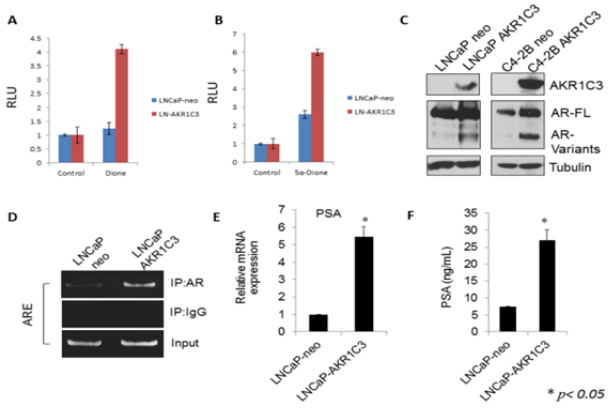
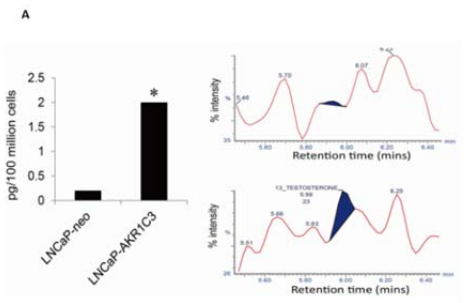


Figure 3. PSA-Luc assays for LNCaP-AKR1C3 cells with or without substrates androstenedion (A) or 5α-dione (B). C. Confirmation of AKR1C3 overexpression; D. ChIP assay for AR binding; and PSA measurements by q-PCR (E) and ELISA (F).

would benefit from overexpression of AKR1C3 and partially overcome the ABI and Enza inhibition (**Figure 2B**). When using the straight AKR1C3 overexpresser LNCaP-AKR1C3, enhancement of transcriptional drive with the reporter construct PSA-Luc was vividly demonstrated by the same pair of pathway intermediates, androstenedione and 5α-dione (**Figure 3A and 3B**). PSA-Luc activities were driven by androstenedione and 5α-dione in LNCaP-AKR1C3 cells grown in charcoal stripped (CS) conditions. Line-up of LNCaP and C4-2B neo and AKR1C3 cells showed AKR1C3 overexpression and increase in AR variants (**Figure 3C**). LNCaP-AKR1C3 cells grown in CS medium were subjected ChIP assay and the result supported AR binding to its androgen response element (ARE) in AKR1C3 overexpressing LNCaP cells compared to the neo control in CS conditions (**Figure 3D**). Both transcript and protein levels of the AR surrogate marker, PSA, were significantly enhanced in LNCaP-AKR1C3 cells with *p* value less than 0.05 (**Figure 3E and 3F**).

Measureing intracellular and intratumor androgen levels by LC-MS induced by AKR1C3 (led by Dr. Gao and assisted by Dr. Evans' labs)



Metabolites (pg/100 million cells)	LNCaP-neo	LN-AKR1C3
Cholesterol	0.0	0.0
etiocholanolne glurcuronide	0.0	16.8
Coprostanol-7,12-diol	0.0	14834.3
27-OH-Cholesterol	14.5	67.4
24-Hydroxycholesterol	214.4	226.1
5a,6a-epoxycholesterol	0.0	57.3
Cholesterol-3-SO4	43955.4	68104.5
Taurocholanic acid	0.9	4.4
Cholesterol-3-Glu	103.8	172.3
Pregnenolone	0.0	0.0
Progesterone	6.5	24.1
20a-Hydroxy-5a-pregnan-3-one	0.0	49.3
17-OH-Pregnanolone-3-Glu-Na	0.0	173.1
Aldosterone	0.0	183.8
Androstenedione	0.0	0.0
Testosterone	0.2	2.1
Dihydrotestosterone	0	0
Etiocholanolone-3-Glu	0.0	288.4
Cortexone	0.0	0.0
Corticosterone	21.1	0.0

Figure 4. Steroids in LNCaP neo and AKR1C3 cells by LC-MS measurement. A. Testosterone; B. detailed lineups of various steroids detected in neo and AKR1C3 cells.

Due to the leakiness of the tet-inducible AKR1C3 line, we relied more on the LN-AKR1C3 and C4-2B-AKR1C3 cells to study the intracrine androgen biosynthesis and animal studies.

One hundred millions (1×10^8) of LNCaP neo and LNCaO-AKR1C3 cells were harvested before maintained in serum and phenol-red free medium for 3 days. Steroids were extracted from cell pellets with 50% methanol and the supernatants were lyophilized followed by subsequent reconstitution to lower the total volume for LC-MS analysis. LNCaP-AKR1C3 cells expressed a significant higher level of testosterone than the vector-control line (**Figure 4A**). The level of progesterone, an intermediate in the androgen biosynthesis pathway, was elevated by almost four fold in the AKR1C3 cells (**Figure 4B**). These results echoed with the enhanced PSA expression in LNCaP-AKR1C3 cells in **Figure 3E and 3F**. C4-2B AKR1C3 cells were harvested in the

Metabolites (pg/mg tissue)	C4-2B parental tumors	C4-2B MDVR tumors
Cholesterol	10626.1	14995.4
7-keto-25-OH cholesterol	493.8	637.4
Coprostanol-7,12-diol	0.0	0.0
27-OH-Cholesterol	0.0	1777.6
24-Hydroxycholesterol	660.9	9485.3
5a,6a-epoxycholesterol	8941.0	6803.1
Cholesterol-3-SO4	3500731. 6	1695625 .7
Taurocholanic acid	1163.2	1911.8
Cholesterol-3-Glu	7916.2	4293.3
5a-Dihydroprogesterone	883.2	974.7
20-Hydroxypregnenolone	2035.4	1035.3
20a-Hydroxy-5a-pregnan-3-one	3966.3	615.2
20a-Dihydroprogesterone	111.1	91.8
PREGNENOLONE	0.0	62.1
Progesterone	1005.7	1043.8
17-HYDROXYPROGESTERONE	58.5	22.0
TESTOSTERONE SULFATE	8.9	0.0
Testosterone GLUC	1218.3	343.5
Etiocholanolone-3-Glu	7551.9	7835.9
DHEAS	49.6	49.1
ALDOSTERONE	0.0	0.0
ANDROSTENEDIONE	194.7	142.7
TESTOSTERONE	1045.9	3458.6
ALLODIHYDROTESTOSTERONE	152.8	240.3
Estrone	295.0	452.5
ESTRADIOL	482.0	373.9
ESTRONE-SO4	42.4	58.9
ESTRONE GLU	0.0	159.4
4-OH ESTRONE	5842.9	15323.1
2-Methoxy-3 OH-estradiol	0.0	19.2
CORTEXONE	164.4	199.0
CORTICOSTERONE	19710.0	11398.3

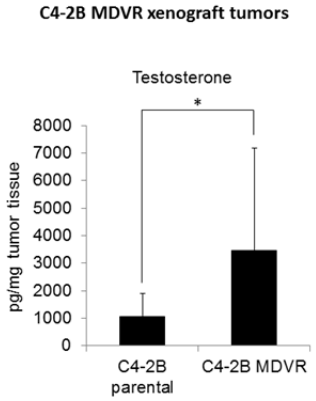


Figure 5. Detailed lineups of various steroids detected in C4-2B parental and MDVR cells by LC-MS measurement.

same fashion together with the neo cells for lysate collection subjected to the intracrine androgen measurement by LC-MS analysis. The samples are currently processed in the West Coast Metabolic Center, UCD. However, we were inspired by the intracrine androgen data from xenograft tumors from C4-2B and C4-2B MDVR orthotopic models. The testosterone level in the MDVR tumors was 3 times higher than the parental one (**Figure 5**). Moreover, there was a significant change in the

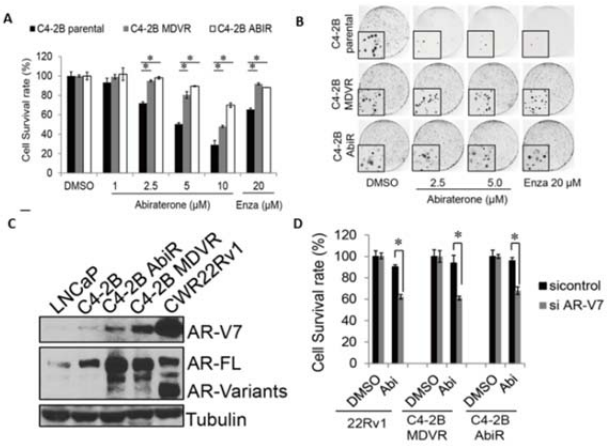


Figure 7. Cross-resistance of C4-2B MDVR and AbiR cells to Abi and Enza by growth (A) and clonogenic (B) assays. C. Levels of AR-V7 and variants in general were higher than the parental lines and similar to CWR22Rv1 cells D. Kinockdown of AR-V7 resensitized cells to Abi.

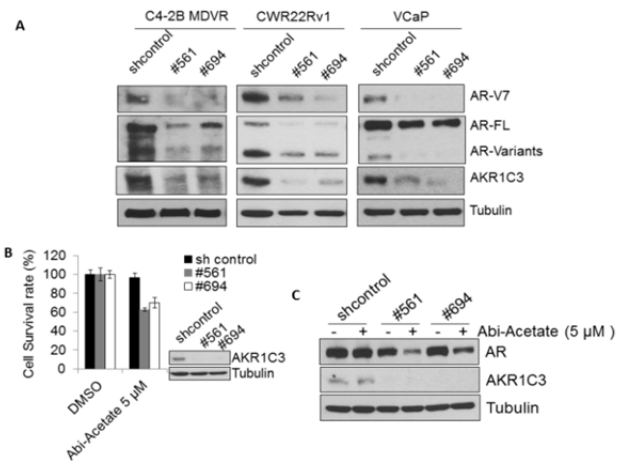


Figure 8. A. shRNA knockdown of AKR1C3 in C4-2B MDVR, CWR22Rv1 and VCaP cells. B. shRNA knockdown of AKR1C3 in C4-2B AbiR cells resensitized the Abi acetate treatments; and C. validation of the knockdown.

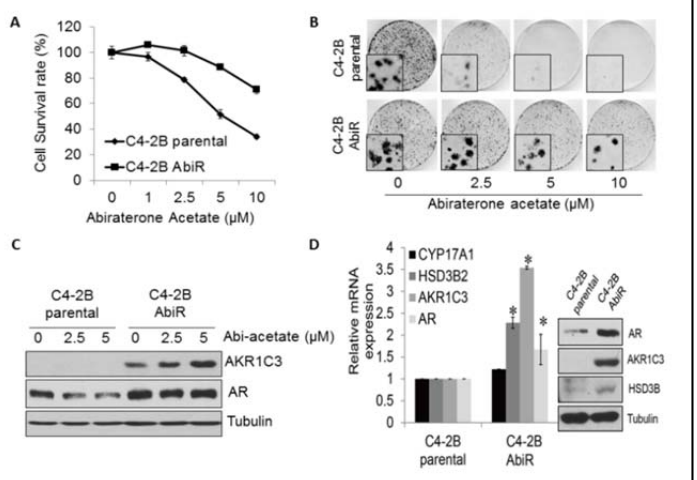


Figure 6. Cell survival curves (A) and clonogenic assays (B) of C4-2B parental and AbiR cells in response to various concentrations of Abi acetate; Characterization of C4-2B AbiR cells in the presence of Abi acetate (C) or in comparison with other lines (D); Examination of other steroidogenic enzymes in AbiR cells by qPCR and Western blotting.

pregnenolone level. Being the immediate product after cholesterol in the steroidogenic pathway, pregnenolone is the main substrate for CYP17A1, target for Abi. We therefore characterized another drug resistant line from C4-2B cells, C4-2B AbiR cells.

C4-2B AbiR cells were resistant to Abi acetate in a dose-response manner compared to the parental line (**Figure 6A**). This result was also confirmed by the clonogenic assay (**Figure 6B**). Abi acetate significantly inhibited the number of colonies in C4-2B parental cells in a dose-dependent manner compared with C4-2B AbiR cells. C4-2B AbiR cells readily expressed increasing levels of AKR1C3 responding to Abi acetate treatments (**Figure 6C**). In addition of AR, variants and AKR1C3, some other steroidogenic enzymes such as CYP17A1 and HSD3B2 also displayed elevation in C4-2B AbiR cells in the mRNA and/or protein levels (**Figure 6D**). Testosterone level in C4-2B AbiR cell was 12 pg/50 million cells, similar to that in C4-2B MDVR or LNCaP-AKR1C3 cells. With the single drug resistant cell lines on hand, we tested for their cross-resistance to Enza and Abi. While the parental line was sensitive to both drugs, the resistant lines showed cross resistance to the other drug at an extent

as depicted in both growth and clonogenic assays (**Figure 7A and 7B**). AR-V7 and variant levels in both MDVR and AbiR cells were detectable together with enhanced AR full-length and variants in general, when compared to those in the parental and LNCaP cells (**Figure 7C**). Knocking down AR-V7 with the specific siRNA resensitized both MDVR and AbiR cells to Abi (**Figure 7D**). To validate the role of AKR1C3 in regulating the leves of AR full-length and variants, we used two small hairpin RNA (shRNA) specific to AKR1C3 to knock down the expression. In C4-2B MDVR or known CRPC such as CWR22Rv1 and VCaP cells, shRNA #561 and #649 both successfully reduced AKR1C3 and drastically decreased AR-V7 levels.in all three cell lines **(Figure 8A)**. Concomitantly, AR full-length protein was also compromised in both C4-2B MDVR and CWR22Rv1 cells. Knocking down AKR1C3 in the AbiR cells resensitized them to Abi acetate treatments and decrease the full-length AR levels. (**Figures 8B and 8C**).

Orthotopic model of LNCaP-AKR1C3 tumor with Enza treatments (led by Dr. Evans' and assisted by Dr. Gao's Labs)

We next tested whether ARK1C3 overexpression would promote the aggressiveness and drug resistanced of PCa tumors in an orthtopic setting. Before conducting the animal study, we propagated LNCaP neo and AKR1C3 cells in CS medium over time and confirmed that LNCaP-AKR1C3 cells proliferated twice faster than the parental ones (**Figure 9A**). Two millions of LNCaP-AKR1C3 cells were mixed with matrigel and injected into the prostate of male SCID mice. When the serum PSA level reached 5-10 ng/ml, mice were surgically castrated. The animals were divided into two groups 2 weeks post castration to be treated with 25 mg/kg of Enza or buffer only (M-F, p.o). Tumor progression was continually monitored by serum PSA and mice were sacrificed after 3 weeks of treatments (**Figure 9B**). Tumors were harvested and compared. Throughout the course, LNCaP-AKR1C3 tumors continued to grow in castrated mice with increased serum PSA. Enza treatments did not prevent tumor progression; there was no difference between tumors from the control and treatment groups visually or by weight (**Figures 9C and 9D**). LC-MS measurement of the intracrine androgens in these tumors is underway.

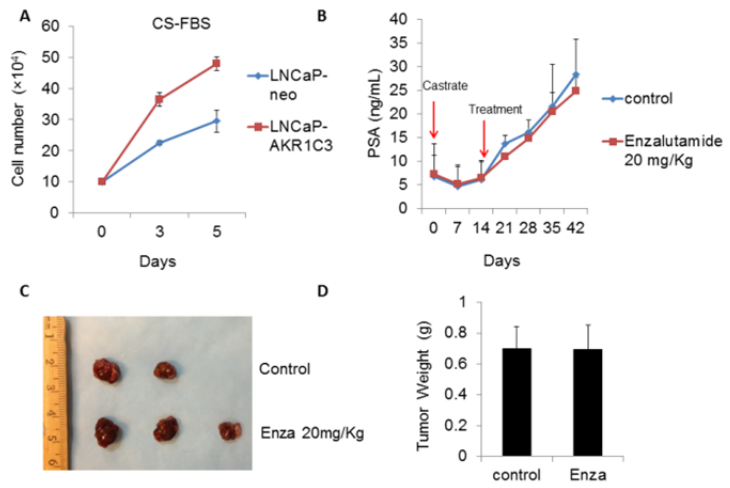


Figure 9. A. Growth curves of LNCaP neo and AKR1C3 cells in CS medium; B. serum PSA monitoring of tumor progression; C. representative tumor pictures and D. tumor weight of control and Enza treated groups.

In summary, we have accomplished all the tasks with teamwork for the first funding year. We generated tet-inducible LNCaP/TR/AKR1C3 cells. We with the help of our collaborator performed the measurement of intracrine androgens in AKR1C3 overexpressing cells. We also used another drug-resistant line, C4-2B AbiR cells to confirm the important role of AKR1C3 in drug resistance possibly through up-regulating AR-V7.

In the next reporting period, we will determine whether blocking AKR1C3 expression will inhibit intraprostatic androgen biosynthesis with both *in vitro* cells and *in vivo* animal (orthotopic) models.

Publications, conference papers, and presentations

1. Liu C, Armstrong CM, Lou W, Lombard A, Evans CP, Gao AC. Inhibition of AKR1C3 Activation Overcomes Resistance to Abiraterone in Advanced Prostate Cancer. Mol Cancer Ther, 2017,16(1): 35-44.

2. Chengfei Liu, Wei Lou, Joy Yang, Chong-Xian Pan, Marc Dall'Era, Christopher Evans, Allen Gao. Targeting AKR1C3 Activation by Indomethacin Overcomes Resistance to Enzalutamide and Abiraterone. The Journal of Urology, 197(4) e771: Abstract # MP57-19.

3. Chong-xian Pan, Chengfei Liu, Primo Lara, Christopher P. Evans, Marc Dall'Era, Stanley Yap, Wei Lou, Allen Gao. Indomethacin to inhibit AKR1C3 intracrine androgen production and sensitizes prostate cancer (PCa) to enzalutamide. Journal of Clinical Oncology, 35:6_suppl, 161-161.

4. Indomethacin inhibits AKR1C3 intracrine androgen production and sensitizes prostate cancer to enzalutamide, Poster, 2017 American Society of Clinical Oncology (ASCO) Annual Meeting.

5. Targeting AKR1C3 activation by indomethacin overcomes resistance to enzalutamide and abiraterone in prostate cancer, Poster, 2017 AUA Annual Meeting.

4. IMPACT

Our study confirmed that up-regulation of AKR1C3 contributed to resistance to anti-androgen therapies. Artificial overexpression of AKR1C3 enabled cell lines to adapt to be drug resistant and to synthesize intracellular and intratumoral androgens.

5. CHANGES/PROBLEMS

Nothing to report.

6. PRODUCTS:

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

(1) Christopher P Evans, MD (PI) No change

(2)

Name:	*Joy Yang, PhD*
Project Role:	*Research Associate*

Researcher Identifier (e.g. ORCID ID):	663002459
Nearest person month worked:	*5*
Contribution to Project:	*Dr. Yang as the main personnel in Evans' lab had participated in all work reported and is the lead person in the orthotopic animal model study.*
Funding Support:	N/A

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to Report.

What other organizations were involved as partners?
Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. **A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site**